Journal of Molecular Diagnostics, Vol. 9, No. 2, April 2007

DOI: 10.2353/jmoldx.2007.060112

Evaluation of the Cepheid GeneXpert *BCR-ABL* Assay

Zsolt Jobbagy,* Reuel van Atta,† Kathleen M. Murphy,* James R. Eshleman,* and Christopher D. Gocke*

From the Molecular Diagnostics Laboratory, Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland; and Cepheid,† Sunnyvale, California*

Patients with chronic myeloid leukemia harbor the chromosomal translocation t(9;22), which corresponds to fusion of the *BCR* and *ABL* genes at the DNA level. The translated fusion product is an oncogenic protein with increased *ABL* tyrosine kinase activity causing cell transformation. To date, reverse transcriptase-polymerase chain reaction is considered the most sensitive method available for detecting low copy numbers of the *BCR-ABL* gene fusion. Recently, Cepheid introduced its GeneXpert-based assay for the identification of the *BCR-ABL* gene fusion in cells from blood samples. This system comprises a walk-away self-contained instrument that combines cartridge-based microfluidic sample preparation with reverse transcriptase-polymerase chain reaction-based fluorescent signal detection and *BCR-ABL* and *ABL* Ct (threshold cycle) determination. The difference between the *BCR-ABL* Ct and *ABL* Ct (ΔCt) is expected to represent the ratio of the two populations of mRNAs and ultimately the percentage of neoplastic cells present. We tested whether this *BCR-ABL* fusion detection system could be used as a clinical diagnostic tool for monitoring patients with minimal residual disease of chronic myelogenous leukemia. We report similar performance characteristics, including limit of detection, specificity, sensitivity, and precision, of this automated *BCR-ABL* fusion detection system to those of a manual TaqMan reverse transcriptase-polymerase chain reaction-based test. ***(J Mol Diagn 2007, 9:220–227; DOI: 10.2353/jmoldx.2007.060112)***

Patients with chronic myeloid leukemia (CML) invariably harbor a chromosomal translocation that corresponds to fusion of the *BCR* and *ABL* genes at the DNA level. The translated fusion product is an oncogenic protein with increased ABL tyrosine kinase activity causing neoplastic transformation. Discovery of this molecular pathway has resulted in more accurate diagnosis, the advent of targeted drug therapy with imatinib mesylate (Gleevec),[1] and, most recently, the application of molecular methodologies for post-therapy follow-up for the presence of minimal residual disease. Minimal residual disease is generally defined as persistence of low numbers of neoplastic cells despite the absence of histological evidence and clinical signs and/or symptoms of the disease.[2] There are many laboratory techniques available to detect and quantify minimal residual disease, including standard cytogenetics, fluorescence *in situ* hybridization, and polymerase chain reaction (PCR)-based detection of residual malignant cells.[3]

Classic cytogenetics (chromosome G-banding) is still considered essential to establish a new diagnosis of CML. In addition to its high specificity in detecting the presence of the t(9;22) translocation, this technique provides the benefit of uncovering other chromosomal abnormalities, thus allowing for more reliable prognostication. Generally, the utility of chromosome banding for monitoring minimal residual disease is limited by the need for satisfactory cell culture for visualization of the metaphases.[4] Furthermore, the relatively low number of cells examined (usually 20 metaphases) results in sensitivities similar to those of the routine histological examination of the bone marrow for the presence of leukemic cells (5%).[5] Sensitivity can theoretically be increased by about 10-fold (to 0.5% leukemia cell content detection) by using fluorescence *in situ* hybridization and examining 200 white blood cells (WBCs); however, the practical sensitivity of interphase fluorescence *in situ* hybridization is only about 1%.[6]

To date, reverse transcription-polymerase chain reaction (RT-PCR) is considered the most sensitive technique available for detecting low copy numbers of the *BCR-ABL* gene fusion, and the combination of RT-PCR with real-time fluorescence detection allows for reliable quantitation of the fusion mRNA. The reported sensitivity for the RT-PCR method varies, but it is estimated at one leukemia cell out of 10^5 to 10^6 normal white blood cells.[7] There are several "home-brew" and commercially available RT-PCR-based *BCR-ABL* detection methodologies used by molecular diagnostic laboratories, each requiring internal validation for the specific laboratory and giving rise to laboratory-specific data. Despite some earlier standardization efforts,[8,9] the

Materials and equipment provided by Cepheid.

Accepted for publication October 4, 2006.

Address reprint requests to Christopher D. Gocke, M.D., Molecular Diagnostics Laboratory, Department of Pathology, Johns Hopkins Medical Institutions, 600 N. Wolfe St., Park SB 202, Baltimore, MD 21287. E-mail: cgocke1@jhmi.edu.

apparent lack of consensus on standardization makes interlaboratory correlation of data difficult.

Monitoring of minimal residual disease in CML using RT-PCR-based molecular techniques confers the ability to assess initial response in patients undergoing drug therapy or after bone marrow transplantation. It also can alert physicians of potential relapses even in cases with complete cytogenetic remission. This information is invaluable to adjust treatment plans and bears important prognostic significance.[10,11] Moreover, it has recently been established that the degree of molecular response at the time of or after achieving complete cytogenetic remission is an independent prognostic factor for progression-free survival.[12]

Cepheid recently introduced its GeneXpert-based assay for the identification of leukemia cells harboring the *BCR-ABL* gene fusion from blood samples. This self-contained automated instrument integrates microfluidic sample preparation with RT-PCR-based, real-time fluorescent signal detection.[13] Virtually all of the reagents used for both the RNA preparation and the subsequent RT-PCR and PCR steps are lyophilized into a disposable multichambered cartridge, potentially decreasing both reagent- and pipette-related bias. The sample is transferred through the chambers during the extraction process by the pumping action of a central piston; subsequently, the purified RNA reaches the detection chamber where the reverse transcription, the multiplexed amplification steps, and the fluorescent signal detection take place. The difference in the measured *BCR-ABL* and *ABL* Ct (threshold cycle) is calculated by the instrument's software and interpreted as positive, negative, or invalid. This difference between the *BCR-ABL* Ct and *ABL* Ct (ΔCt) is expected to represent the ratio of the two populations of mRNAs and ultimately the fraction of neoplastic cells present.

We tested whether this new *BCR-ABL* fusion detection system could be an alternative to current clinical diagnostic tools for monitoring CML patients for minimal residual disease. We have compared patient test results obtained with this new cartridge-based system and with our clinical analyte specific reagent (ASR) test to define specificity, sensitivity, and correlation between the two methods using standard curves based on plasmid DNA standards. We have also compared additional performance characteristics, including limit of detection and precision for the two systems using normal blood samples spiked with either K562 cells or *BCR-ABL* Armored RNA.

Materials and Methods

The Johns Hopkins Medicine Institutional Review Board approved this study. We tested 53 patient peripheral blood samples by both methods. The negative control group included 13 blood samples. These were obtained from five healthy bone marrow donors and eight patients with hematological disorders unrelated to *BCR-ABL* gene fusion (two with acute lymphoblastic leukemia, two with Hodgkin lymphoma, one with acute myelogenous leukemia, one with mucosa-associated lymphoid tissue lymphoma, one with multiple myeloma, and one with follicular lymphoma). The remaining 40 clinical samples received for the clinical ASR *BCR-ABL* testing belonged to 39 patients with an established diagnosis of CML and one patient with Philadelphia chromosome-positive acute lymphoblastic leukemia.

Fresh blood samples, donated by a healthy volunteer, were confirmed as negative for *BCR-ABL* fusion by both assays and were used as matrix for the spiking experiments. Automated white blood cell counts were obtained for all three venipuncture samples [mean WBCs = 6.39 ($\pm$0.22) $\times$ 1000/μl]. GeneXpert runs, as well as the RNA isolation protocol for the reference assay, were always performed within 24 hours from the time of venipuncture, the blood samples were kept refrigerated, and the RNA samples were stored at $-$80°C to minimize the possibility of RNA degradation.

All samples were tested in duplicate using both the cartridge-based system and the clinical ASR assay. Samples with ambiguous results on the cartridge-based tests (positive-negative, invalid-positive, invalid-negative) were subjected to a third run, and the consensus result was used.

GeneXpert cartridges and reagent solutions (proteinase K, lysis buffer, wash buffers 1, 2, and 3) as prepacked kits and in bulk were provided by Cepheid (Sunnyvale, CA). The M-*BCR* Fusion*Quant* kit and the *ABL* and *BCR-ABL* plasmid standards (Fusion*Quant* Standard) were purchased from Ipsogen (Marseille, France). The b2a2 Armored RNA standards were generously provided by Ambion (Austin, TX).

K562 cells (ATCC) were grown in Iscove's Dulbecco's modified medium containing 10% fetal calf serum and 1.5 g/l L-glutamine (Gibco BRL, Gaithersburg, MD) at 37°C, 5% CO_2 atmosphere. The K562 cells were harvested by centrifugation (5000 rpm for 5 minutes) at room temperature and resuspended in ice-cold phosphate-buffered saline. Automated cell counts were obtained for both the K562 cells and the blood samples, and aliquots containing K562 cells were mixed into 2400 μl of fresh blood. Three 200-μl aliquots were taken for the GeneXpert assays, and the remaining blood was subjected to the RNA purification protocol for the Fusion*Quant* assay. For the lowest K562 cell number samples, the 200-μl blood aliquots were individually spiked with five or 12 K562 cells to ensure even distribution of the neoplastic cells. On the high neoplastic cell number end of the experiment, we substituted parts of the 200-μl volume of normal blood with phosphate-buffered saline. This ensured that in the samples spiked with 120,000 and 1,200,000 K562 cells, the overall WBC number remained constant, and failed runs could be avoided. For example, we have noted that at very high white blood cell counts (eg, 200-μl samples with over 190,000 WBC counts) repeated runs resulted in error messages, whereas 10-fold dilution of these samples with phosphate-buffered saline led to valid runs. Likewise, fresh blood was spiked with aliquots of Armored *BCR-ABL* RNA (b2a2). The GeneXpert assays were run according to the manufacturer's protocol. Briefly, 200 μl of whole blood was mixed with 40 μl of

proteinase K and incubated at room temperature for 1 minute. Subsequently, 1000 μl of lysis buffer was added, mixed by vortexing for 10 seconds, and incubated at room temperature for 10 minutes. One thousand microliters of 100% ethanol was added, mixed by vortexing for 10 seconds, and loaded into the cartridges.

The Fusion*Quant* Kit (FQPP-10 M-BCR) clinical ASR assay was run according to our standard laboratory protocol. The kit contained a primer/probe mix and standards for the *BCR-ABL* fusion. A primer/probe mix and standards for *ABL* were used as control. Briefly, total RNA was extracted from 1.5- to 3-ml volumes of the peripheral blood samples using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA). A final volume of 25 μl of reaction mix containing QuantiTect Probe RT-PCR Master mix, primer/probe mix, and QuantiTect RT mix and 300 ng of RNA was distributed in 96-well plates. The plates were loaded and run on a TaqMan 7900 real-time thermal cycler (Applied BioSystems, Foster City, CA). For each run, *BCR-ABL* and *ABL* plasmid DNA-based standard curves were generated, and actual copy numbers were determined for both the *BCR-ABL* fusion gene and the *ABL* gene. The ratio of the two populations was reported as [*BCR-ABL* copies/1000 *ABL* copies] unless noted otherwise. The data were analyzed and graphed using Microsoft Excel (Microsoft, Redmond, WA) and MedCalc (MedCalc Software, Mariakerke, Belgium) software.

Results

Relationship between BCR-ABL and ABL Plasmid Copy Numbers and Cycle Threshold (Ct) Values

To compare directly the performance characteristics of the novel cartridge-based system to those of our clinical ASR assay, we established standard curves for both methods using the same serially diluted plasmid DNA standards. The clinical ASR assay determines *BCR-ABL* and *ABL* copy numbers for a given sample separately by using equations obtained from Ct versus *ABL* copy number and Ct versus *BCR-ABL* copy number standard curves, respectively (Figure 1, A and B, solid lines). The ratio of [*BCR-ABL* copies]/[1000 *ABL* copies] is reported for clinical purposes. To directly compare the two systems, we generated standard curves for the cartridge-based assay (Figure 1, A and B, dashed lines). The GeneXpert cartridges contain primer pairs and probes that would simultaneously detect the *BCR-ABL* fusion sequence and the spliced a2-a3 *ABL* gene region of a fusion product within the same reaction.[14] This design produces an in-tube control to compare the efficacy of the *BCR-ABL* fusion region detection and the detection of the *ABL* gene portion of the same *BCR-ABL* Fusion*Quant* plasmid standard. Regression analysis showed that the regions were amplified with similar efficiency, showing a slope of 1.018 and *y* intercept of 0.0685 ($R^2 = 0.9961$) for the plot (Figure 1D). Regression analysis comparing the average Ct values obtained for each Fusion*Quant* plasmid concentration (*BCR-ABL* and *ABL* copy numbers) by both the cartridge-based and the clinical ASR assays showed a linear relationship between performances of the two methods over the studied copy number ranges with coefficients of determination greater than 0.99 (Figure 1C).

Determination of Linear Range and Limit of Detection for the GeneXpert Assay Using K562 Cell Spiked Blood Samples

The K562 cell line was originally cultured from the pleural effusion of a patient with terminal blast crisis of CML and harbors a b3a2 type *BCR-ABL* fusion product.[15] To determine the relationship between neoplastic cell number and ΔCt (*BCR-ABL* Ct − *ABL* Ct) in an environment most similar to clinical samples, we spiked different numbers of K562 cells into normal blood as described in Materials and Methods. Two hundred-microliter aliquots of these samples were run on the cartridge-based system in duplicates or triplicates in two separate experiments. The data were combined, and ΔCt values calculated and plotted against K562 cell numbers (Figure 2A, diamonds). At the lowest dilution (five K562 cells/200 μl of blood) only one of three replicate runs yielded a positive result. To determine the limit of detection, 21 samples (200 μl of blood spiked with 12 K562 cells/each sample) were tested with the cartridge-based system within 24 hours of venipuncture. Twenty samples tested positive, and one sample tested negative, establishing the limit of detection for this assay at the level of 12 cells/200 μl of blood (12 K562 cells/1.2 million WBCs) with a 95.2% confidence. At extreme K562 cell numbers (120,000 and 1,200,000), duplicate runs showed high precision, but the ΔCt values appeared to be skewed (1.7 ± 0.14 and 1 ± 0, respectively), because a positive control run of K562 cells only (without normal blood matrix) resulted in a ΔCt value of 1.5, representing the highest possible *BCR-ABL* to *ABL* ratio (and lowest possible ΔCt value) for any mixture of K562 cells and normal WBCs. The ΔCt values in the range of 12 to 12,000 K562 cells/200 μl of blood showed linearity on a semilogarithmic scale with a good fit ($R^2 = 0.9918$) (Figure 2B, dashed line).

The same K562 cell-spiked samples were processed for the clinical assay performed with the reference *BCR-ABL* fusion detection kit. Although we calculated and plotted ΔCt values for each spiked sample (Figure 2A, squares), the Ct values for *BCR-ABL* and *ABL* were measured in independent reactions so that ΔCt values obtained by the clinical ASR test exhibit a potential systemic error absent from the GeneXpert system. This may have contributed to the presence of two outliers (at five and 50 K562 cells) and the calculated negative ΔCt values at high K562 cell numbers. The results obtained by both methods were expressed as the ratio of *BCR-ABL* fusion mRNA copy number and *ABL* mRNA copy number (*BCR-ABL* copies/10,000 *ABL* copies) and were correlated (Figure 2C). Regression analysis suggested similar performance of the two assays ($y = 1.2756 + 0.9862x$, $R^2 = 0.9642$, $r = 0.9819$, $P < 0.0001$ calculated with the omis-

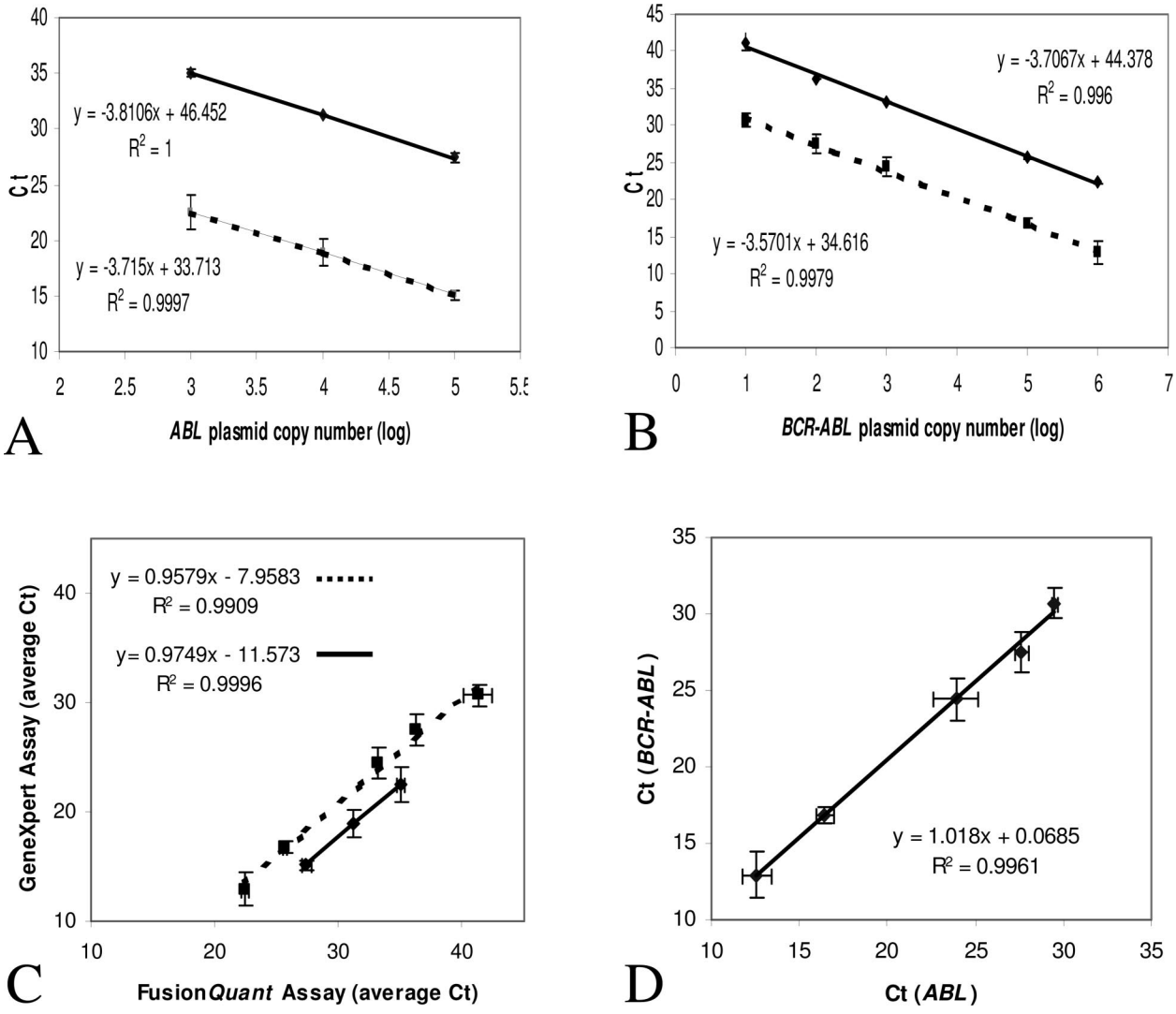

Figure 1. Fusion*Quant* plasmid copy number-based standard curves. **A** and **B:** Relationship of average Ct values to Fusion*Quant* Standard plasmid copy numbers obtained by the GeneXpert (dashed line) and clinical ASR (solid line) methods. **C:** Correlation between the GeneXpert and the clinical ASR assays using average Ct values obtained for *ABL* (solid line) and *BCR-ABL* (dashed line) Fusion*Quant* plasmid standards. **D:** Comparison of the efficiencies of the *BCR-ABL* and *ABL* reactions within the same GeneXpert multiplex PCR assays. For the GeneXpert method, each data point represents the average of three independent runs (except for the 10-copy data point shown on graph **B**, where two positive and three invalid results were obtained out of five runs). For the reference method, each data point represents the average of two independent assays as described in our standard operating protocol for the clinical assay.

sion of the Fusion*Quant* assay-related outlier at 50 K562 cell number).

Determination of Precision for the GeneXpert System and the Clinical ASR Assay

To determine the coefficient of variation (CV) value for the GeneXpert system, we ran eight 200-μl samples of fresh normal blood containing 5000 copies of Armored RNA (b2a2 fusion) each. The resulting Ct values were used to calculate *BCR-ABL* and *ABL* copy numbers using the respective equations established for the plasmid-based standard curves. The replicate copy number values were averaged and the ratios of *BCR-ABL* copy numbers to *ABL* copy numbers were calculated and recorded in the [*BCR-ABL*/1000 *ABL*] form. The CV value for the reference assay was similarly calculated using the ratios of [*BCR-ABL* copy numbers] and [1000 *ABL* copy numbers], obtained for the same aliquot of a positive control RNA sample assayed 16 times. The resulting CV values were similar for the two assays (40.2 and 42.5%, respectively).

*Sensitivity, Specificity, and Correlation between the GeneXpert and the Fusion*Quant *Assays Based on Analysis of Clinical Samples*

We tested 53 patient peripheral blood samples by both the reference clinical ASR and the GeneXpert methods. The negative control patient group included five healthy bone marrow donors, two patients with acute lymphoblastic leukemia, two with Hodgkin's lymphoma, one with acute myelogenous leukemia, one with mucosa-associ-

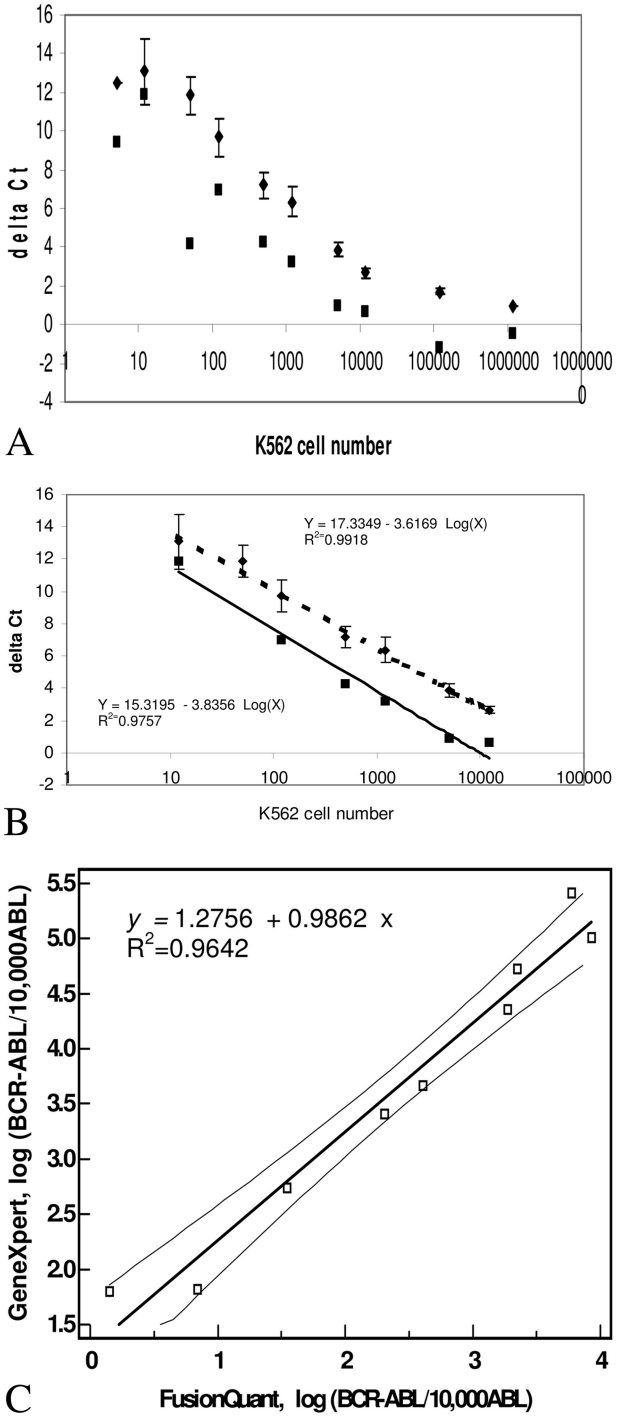

Figure 2. K562 cell number-based standard curves. **A:** Relationship between K562 cell numbers and calculated ΔCt values obtained by the two assays. For the GeneXpert assay (**diamonds** and **dashed line**), each data point represents the average of two to four independent runs obtained in two separate experiments except for the lowest K562 cell number,[5] which represents the single positive result out of three valid runs. For the Fusion*Quant* assay (**squares** and **solid line**), each data point represents the average of two independent runs obtained from two separate experiments except for K562 cell numbers 5, 50, 500, and 5000, where only single measurements were possible due to limited samples. **B:** Linear regression plots of the calculated ΔCt values within 12 to 12,000 K562 cell number range for the GeneXpert (dashed line) and Fusion*Quant* (solid line) assays. The outlier at 50 K562 cell number was omitted for the Fusion*Quant* assay plot. **C:** Linear regression plot for comparison of the GeneXpert and the clinical ASR methods. One outlier related to the Fusion*Quant* assay was omitted.

ated lymphoid tissue lymphoma, one with multiple myeloma, and one with follicular lymphoma. The remaining 40 clinical samples were obtained from patients with established diagnoses of CML (39 patients) or Philadelphia chromosome-positive acute lymphoblastic leukemia (one patient).

All 13 negative control samples tested negative by both methods (100% specificity for both methods), and an additional six clinical samples proved negative by the clinical ASR assay but only five of those by the cartridge-based method, resulting in an overall specificity of 94.7% for the latter method as compared with the reference ASR assay. (Note: The "false-positive" result was obtained from two positive runs with *BCR-ABL* Ct values of 30.5 and 29.8 and one negative run with no *BCR-ABL* signal.)

Thirty-four clinical samples tested positive with our clinical ASR kit. Thirty-one of those were also detected as positive by the cartridge-based assay (91.2% sensitivity). Deming regression analysis[16] of the positive values obtained by both methods confirmed a linear relationship between the two datasets generated by the clinical ASR assay and the GeneXpert method, respectively, and indicated constant bias ($Y = 0.1756 + 0.9995X$) (Figure 3A). A Bland-Altman difference plot[17] was also generated for these datasets, and the distribution of data was consistent with a classic percent difference plot indicating systematic error only (Figure 3B).

Discussion

The prognostic importance of molecular diagnostic monitoring of CML patients is now well established.[10–12,18–20] Quantitative real-time reverse transcription-PCR (qRT-PCR) is the most sensitive method available to detect low copy numbers of the *BCR-ABL* fusion products.[7] The currently used qRT-PCR procedures are highly complex, laborious and, despite some standardization efforts,[8,9] often produce clinical results reportable in different formats for each assay.

Recently, Cepheid adapted its proprietary cartridge-based automated real-time RT-PCR system for the detection of the *BCR-ABL* fusion mRNA. We compared this cartridge-based automated system to determine whether it could be a useful alternative to our current clinical ASR assay for monitoring patients with minimal residual disease of chronic phase CML. Standard curves were generated for both systems using the Fusion*Quant* (M-BCR kit; Ipsogen) plasmid sets, showing linear relationships between copy numbers and *BCR-ABL* Ct or *ABL* Ct on semilogarithmic scales. The equations defining these standard curves were later used to calculate absolute *BCR-ABL* and *ABL* copy numbers for direct comparison of the two assays. These standard curve experiments established that the Ct values obtained by the GeneXpert measurements reliably (similar equations for both *BCR-ABL* and *ABL* standards) and highly correlated (R^2 values over 0.99) with those obtained by the reference assay (Figure 1). It should be noted that the manufacturer provides a lot-specific efficiency value ($E_{\Delta Ct}$) to translate ΔCt to relative copy number for each lot of cartridges.[14] Using

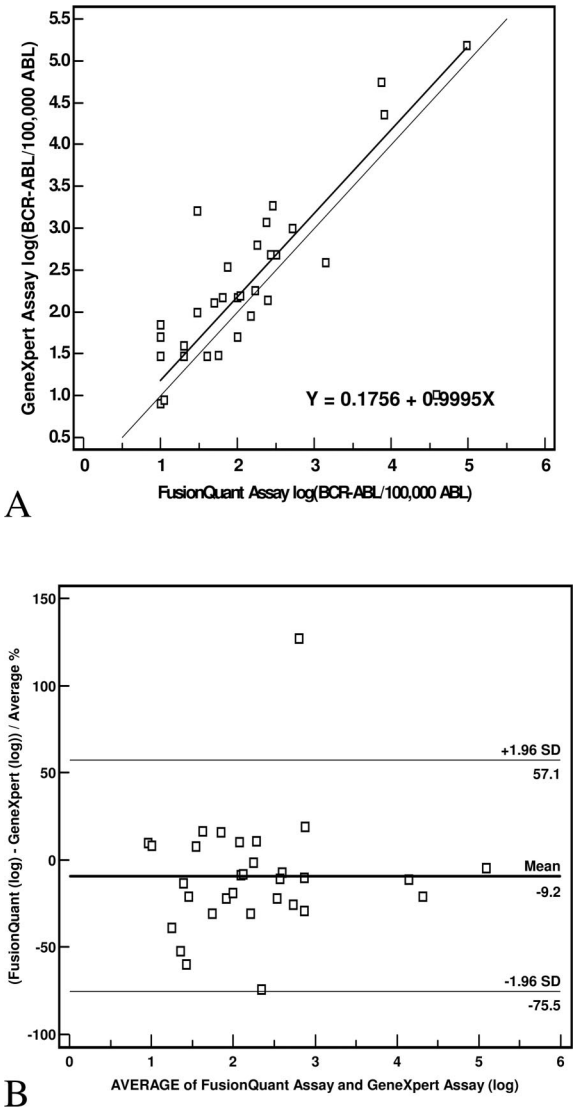

Figure 3. Comparison of the GeneXpert and the reference assay for the quantification of relative ratios of *BCR-ABL* and *ABL* gene products from clinical samples. **A:** Deming regression plot. The solid line corresponds to the regression line and the dashed line represents the identity line. **B:** Bland-Altman percent difference plot.

this number one can calculate the *BCR-ABL/ABL* ratio without resorting to creation of a dilution curve.

K562 cell spiking experiments determined that the limit of detection for the GeneXpert system was 12 neoplastic cells (95.2% confidence) corresponding to the detection of one leukemic cell out of 10^5 white blood cells. This resolution is very similar to those reported for other RT-PCR-based clinical assays.[7,12] The cartridge-based assay showed a linear relationship between ΔCt (*BCR-ABL* Ct − *ABL* Ct) over three logs (between 12 and 12,000 K562 cells/1.2 × 10^6 WBCs). Importantly, detecting and quantifying neoplastic cells in this range is essential for monitoring minimal residual disease.[12] Interestingly, the clinical ASR kit results for the same samples showed larger bias, presumably due to the physically separate reactions for *BCR-ABL* and *ABL* detection. Still, the overall comparison of the ΔCt versus K562 cell number plots for the two systems showed similar slopes and regression analysis indicating a linear relationship between data expressed as relative copy numbers for the two systems (Figure 2). The apparently lower degree of correlation for this spiking experiment as compared with correlation seen in Figure 1 can be explained by the larger biases seen with both assays for more complex biological samples and the logarithmic calculations involved in getting the results in the [*BCR-ABL* copies/1000 *ABL* copies] form. At higher K562 cell numbers (120,000 and 1,200,000, respectively), the results appeared to be distorted with similar or even lower ΔCt values as compared with the ΔCt obtained by testing of K562 cells only (without normal blood matrix), representing the highest possible *BCR-ABL* to *ABL* ratio (and lowest possible ΔCt value) for any mixture of K562 cells and normal WBCs. Goldman predicted this phenomenon, attributable to the high proportion of *ABL* detected from within the *BCR-ABL* fusion product itself, for similar primer-pair designs.[7] Despite this potential drawback, the use of *ABL* as the control gene is supported by data showing the least variation of expression between normal and leukemic samples as compared with the larger expression differences seen for β-glucuronidase and β-2-microglobulin genes.[21] Furthermore, it is highly unlikely to influence significantly the relative quantitation of the fusion products at the low copy numbers seen with minimal residual disease. More recent studies, however, suggest that β-glucuronidase might be a more reliable internal control for *BCR/ABL* quantitation.[22,23]

The GeneXpert system employs four discrete PCR/fluorescence detection bays. In addition, each cartridge serves as a separate device for RNA purification and contains mixtures for complex PCR reactions. For these reasons, each run can be considered completely independent from any other, most similar to runs in day-to-day variation studies conducted for clinical chemistry assays. Comparison of CV values obtained for the two systems showed remarkable similarity (Fusion*Quant* 42.5% and GeneXpert 40.2%), despite the fact that the cartridge-based assay used a less pure substrate (spiked whole blood versus purified RNA in the clinical ASR assay), with potential bias originating from the RNA purification cycles and the variability inherent in each cartridge and each bay of the automated instrument serving as a distinct PCR machine and fluorescence detector. These CV values were based on calculated relative ratios for both systems and represented significantly higher variance compared with those calculated from primary Ct data (*ABL* Ct CVs of 5.2 and 3.5% and *BCR-ABL* Ct CVs of 4.7 and 3.9% for the GeneXpert and Fusion*Quant* methods, respectively). We did not see any difference between cartridges of the two different lots available to us for this study. This finding is in agreement with the experience from a large precision study.[14] The GeneXpert system showed high sensitivity (91.2%) and specificity (95%) as compared with the clinical ASR reference method, based on the simplified result reporting (positive, negative, or invalid) of the system software. The few false-negative samples either yielded no *BCR-ABL* signal (*BCR-ABL* Ct = 0) or were interpreted as negative by the software

due to the preset threshold (*BCR-ABL* Ct must be less than or equal to 32[14]). Further studies may be needed to fine tune criteria for reporting results as negative. The corresponding clinical ASR assay results for these false negatives invariably fell below 0.05 *BCR-ABL* copies/1000 *ABL* copies, which is considered the limit of detection for this assay in our laboratory. A Bland-Altman percent difference plot showed only systematic bias, indicating that the two systems would be interchangeable assuming that the differences within the mean ± 1.96 SD limits are clinically acceptable.

The GeneXpert system has the obvious advantage of near full automation over conventional commercial or home-brew *BCR-ABL* assays. The hands-on assay setup time is less than 30 minutes, including 10 minutes' incubation time (for one to four assays), and the total test time is about 2.5 hours. The lack of need for batch processing (random access) confers the ability for on-demand testing. Another benefit of the system is that most of the chemistries used for both the RNA preparation and the subsequent RT-PCR and PCR steps are lyophilized into the disposable multichambered cartridge, potentially decreasing both reagent-related and human errors. The machine has a simplified interface, reporting results as positive, negative, or invalid but also collects and stores Ct values (*BCR-ABL* Ct and *ABL* Ct), rendering it suitable for more detailed quantitative analysis of the data.

We have established the limit of detection as one K562 cell detected out of 10^5 WBCs for the GeneXpert assay. This means that any samples with more than 500 WBCs/μl should be detectable as positive if containing neoplastic cells at or above the $1{:}10^5$ ratio. Samples with lower than 500/μl WBC counts should not be applied without some preparations to increase WBC concentration. Despite this potential shortcoming of the GeneXpert system, we did not find significant differences in the sensitivity of the two methods using the clinical samples (probably because all of the 54 samples examined had WBC counts above 500). Although the Fusion*Quant* system uses 1.5- to 3-ml blood samples to start with, the input of total RNA into the RT-PCR reaction is optimized around 300 ng. Our total RNA purification method yields approximately 3.8 pg of RNA/K562 cell. The 300 ng of total RNA would thus represent around 80,000 cells in our clinical ASR assay assuming that the total RNA content is similar in K562 cells and in normal WBCs. This would allow for a detection limit of approximately one neoplastic cell in nearly 10^5 cells. Increasing the starting blood volume does not necessarily increase sensitivity in the clinical ASR either. On the other hand, the detection limit of one neoplastic cell out of 10^5 WBCs achieved by the GeneXpert assay corresponds to the above value as well as to the limits of detection reported for other methods.[7,12] In addition, the question of the clinical significance of the least detectable amount of *BCR/ABL* fusion is still not resolved. At the same time, we think that the GeneXpert system in its current form cannot completely replace other conventional methods (like Fusion*Quant*), and those patients with negative GeneXpert results could benefit from reflex testing with Fusion*Quant* or other established methods. The availability of a parallel method is also required by the lack of minor breakpoint *BCR/ABL* detection capability of the GeneXpert system.

By providing faster turnaround times, requiring less hands-on time, fewer technical skills, and offering the possibility of more convenient cross-laboratory standardization, this assay system could provide benefits over currently used home-brew or commercial *BCR-ABL* fusion detection methods. Among the limitations of this cartridge-based assay are that only major breakpoint chemistries are available at this point, and there may be a drop in sensitivity at extremely low copy numbers. Running negative samples in replicate may enhance sensitivity. In summary, our experiments indicate that the GeneXpert *BCR-ABL* assay has similar overall performance characteristics to those of the Fusion*Quant* kit-based ASR clinical assay.

Acknowledgments

We thank Telissa Crevasse and Roy Rich for their expert technical help with the project, and Cepheid and Ambion for providing us with research materials.

References

1. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB: Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996, 2:561–566
2. Butturini A, Klein J, Gale RP: Modeling minimal residual disease (MRD)-testing. Leuk Res 2003, 27:293–300
3. Tefferi A, Dewald GW, Litzow ML, Cortes J, Mauro MJ, Talpaz M, Kantarjian HM: Chronic myeloid leukemia: current application of cytogenetics and molecular testing for diagnosis and treatment. Mayo Clin Proc 2005, 80:390–402
4. Nashed AL, Rao KW, Gulley ML: Clinical applications of BCR-ABL molecular testing in acute leukemia. J Mol Diagn 2003, 5:63–72
5. Gutterman JU, Mavligit G, Burgess MA, McCredie KB, Hunter C, Freireich EJ, Hersh EM: Immunodiagnosis of acute leukemia: detection of residual disease. J Natl Cancer Inst 1974, 53:389–392
6. Pelz AF, Kroning H, Franke A, Wieacker P, Stumm M: High reliability and sensitivity of the BCR/ABL1 D-FISH test for the detection of BCR/ABL rearrangements. Ann Hematol 2002, 81:147–153
7. Goldman J: Monitoring minimal residual disease in BCR-ABL-positive chronic myeloid leukemia in the imatinib era. Curr Opin Hematol 2005, 12:33–39
8. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, van Dongen JJ: Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia—a Europe Against Cancer program. Leukemia 2003, 17:2318–2357
9. Hochhaus A: A further milestone towards comprehensive standardization of quantitative RT-PCR protocols for leukemic fusion gene transcripts has been reached. Leukemia 2003, 17:2383–2384
10. Mughal TI, Yong A, Szydlo RM, Dazzi F, Olavarria E, van Rhee F, Kaeda J, Cross NC, Craddock C, Kanfer E, Apperley J, Goldman JM: Molecular studies in patients with chronic myeloid leukaemia in remission 5 years after allogeneic stem cell transplant define the risk of subsequent relapse. Br J Haematol 2001, 115:569–574
11. Olavarria E, Kanfer E, Szydlo R, Kaeda J, Rezvani K, Cwynarski K, Pocock C, Dazzi F, Craddock C, Apperley JF, Cross NC, Goldman JM: Early detection of BCR-ABL transcripts by quantitative reverse transcriptase-polymerase chain reaction predicts outcome after allo-

geneic stem cell transplantation for chronic myeloid leukemia. Blood 2001, 97:1560–1565
12. Press RD, Love Z, Tronnes AA, Yang R, Tran T, Mongoue-Tchokote S, Mori M, Mauro MJ, Deininger MW, Druker BJ: BCR-ABL mRNA levels at and after the time of a complete cytogenetic response (CCR) predict the duration of CCR in imatinib mesylate-treated patients with CML. Blood 2006, 107:4250–4256
13. Raja S, Ching J, Xi L, Hughes SJ, Chang R, Wong W, McMillan W, Gooding WE, McCarty Jr KS, Chestney M, Luketich JD, Godfrey TE: Technology for automated, rapid, and quantitative PCR or reverse transcription-PCR clinical testing. Clin Chem 2005, 51:882–890
14. Winn-Deen E, Helton B, van Atta R, Peralta J, Wang J, Radich J, Tsongalis G, Belloni D, Chan D, Eshleman J, Jobbagy Z, Gocke C: Development of an integrated assay for quantitative real-time detection of BCR-ABL RNA from peripheral blood. Haematologica 2006, 91:19–20
15. Lozzio CB, Lozzio BB: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 1975, 45:321–334
16. Cornbleet PJ, Gochman N: Incorrect least-squares regression coefficients in method-comparison analysis. Clin Chem 1979, 25:432–438
17. Bland JM, Altman DG: Measuring agreement in method comparison studies. Stat Methods Med Res 1999, 8:135–160
18. Kantarjian HM, Cortes JE, O'Brien S, Luthra R, Giles F, Verstovsek S, Faderl S, Thomas D, Garcia-Manero G, Rios MB, Shan J, Jones D, Talpaz M: Long-term survival benefit and improved complete cytogenetic and molecular response rates with imatinib mesylate in Philadelphia chromosome-positive chronic-phase chronic myeloid leukemia after failure of interferon-alpha. Blood 2004, 104:1979–1988
19. Marin D, Marktel S, Bua M, Szydlo RM, Franceschino A, Nathan I, Foot N, Crawley C, Na Nakorn T, Olavarria E, Lennard A, Neylon A, O'Brien SG, Goldman JM, Apperley JF: Prognostic factors for patients with chronic myeloid leukaemia in chronic phase treated with imatinib mesylate after failure of interferon alfa. Leukemia 2003, 17:1448–1453
20. Wang L, Pearson K, Ferguson JE, Clark RE: The early molecular response to imatinib predicts cytogenetic and clinical outcome in chronic myeloid leukaemia. Br J Haematol 2003, 120:990–999
21. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, Delabesse E, Macintyre E, Gottardi E, Saglio G, Watzinger F, Lion T, van Dongen JJ, Hokland P, Gabert J: Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR)—a Europe Against Cancer Program. Leukemia 2003, 17:2474–2486
22. Lee JW, Chen Q, Knowles DM, Cesarman E, Wang YL: beta-Glucuronidase is an optimal normalization control gene for molecular monitoring of chronic myelogenous leukemia. J Mol Diagn 2006, 8:385–389
23. Wang YL, Lee JW, Cesarman E, Jin DK, Csernus B: Molecular monitoring of chronic myelogenous leukemia: identification of the most suitable internal control gene for real-time quantification of BCR-ABL transcripts. J Mol Diagn 2006, 8:231–239